

22/2/21

→ Lehninger Pg - 705
→ Igenetics Pg - 194

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Transformation is defined as the uptake of exogenous DNA into host cell extracellularly.

- To isolate cell which contain α T cells having α plasmid with GOI (0.01%) we use different enzymes which increase success rate of α T cells.
- Enzyme in RDT which will help to perform experiment easily.

→ Nuclease : 2 types

- 1) DNase - Enzyme which depolymerises DNA.
- 2) RNase - Enzyme which depolymerises RNA

DNase are of 2 types

i] Exonuclease : work at free end of DNA (makes 2 cuts, 3 nucleotide forms)

ii] Endonuclease : Work in between DNA strands
leaves internal phosphodiester bond
MOA (mode of action) [Treating DNA with nuclease]

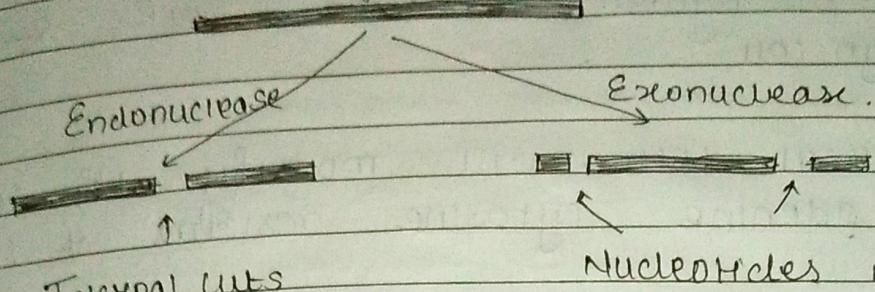
Endonuclease

- breaks entire ds DNA into pieces

Exonuclease

- If ds DNA linear, the exonuclease will remove one nucleotide at a time & entirely degraded into 3 nucleotide.

Activity of nuclease.



Biological function (specific nucleotide-pair sequence in DNA) is to recognize and cleave foreign DNA (DNA = called restricted) → hydrolysed phosphodiester bond.

Prokaryotic cell have restriction system which make use of restriction enzyme (endonucleases) → provide immunity to bacterium against viral infection.

Restriction system can't classify between self & foreign cells.

Consider a bacteriophage which infects E. coli.

- Most of the bacteriophage contain ds linear DNA.
- Few bacteriophage contain ss circular DNA.
- When viral nucleic acid enters the bacterial cell, the bacterial cell will think that it is not good for it and restriction enzyme (endonuclease) will start attacking viral nucleic acid and then they will be broken into fragments.

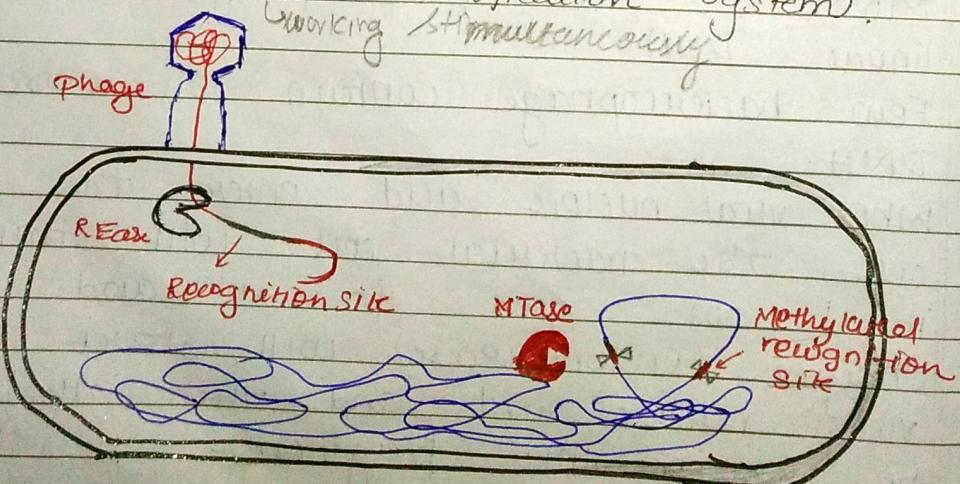
The bacterium modifies its own restriction sites (by methylation) so that its own ^{Page No.} ^{Date} is protected from restriction Enzyme.

Modification System

- Can distinguish between self & foreign cell.
- Bacterial cell will modify its cell by adenine, cytosine residue of its own.
- Bacterial cell possess restriction system (use restriction enzymes) to stop foreign material.
- DNA strand has adenine or cytosine adyega to methylation or glycosylation ↓ noga ^{specific (A and C)}.

Very rare in presence of glucosidase. Will not get affected by the action of any foreign particle.

RMS (Restriction Modification System).



• In host cell's DNA

→ sequence recognized by its own restriction endonucleases is protected from digestion \Rightarrow by methylation of DNA.
catalysed by a specific DNA methylase.

The restriction endonuclease + corresponding methylase are sometimes referred as

\Downarrow

"Restriction - modification System."

Two classes of enzyme approach to generating
and propagating a recombinant

DNA	Molecules
Data	

1] Restriction Endonucleases 2] DNA ligases

Restriction endonucleases / Restriction Enzyme

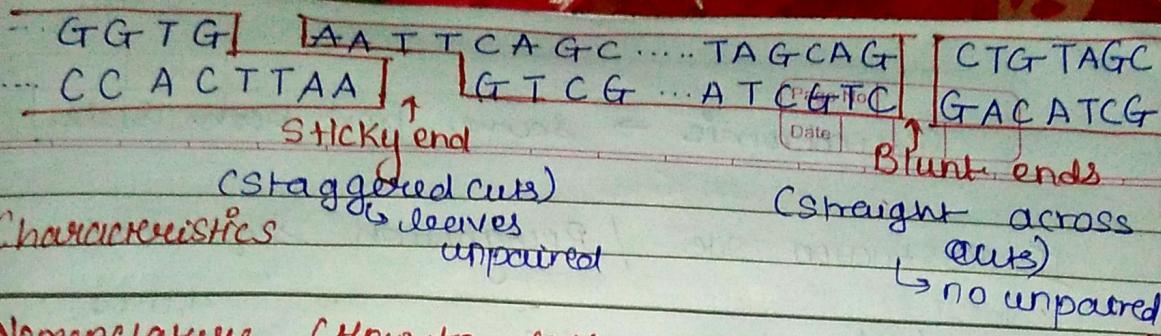
recognize and cleave DNA at specific sequence
(recognition sequence / restriction site) to generate
sets of smaller fragments.

3 Types

- Type I → i cleaves DNA at random sites
- Type II → ii move with DNA in Rn and require the energy of ATP.

Type III → very specific

- Single polypeptide chain easily removed.
- easily purified by column chromatography (20-100 kDa).
- Mg^{+2} require as co-factor only.
Type I, III → ATP, co-factors.
- Extremely specific towards the restriction site
- Cleaves the DNA within the recognition sequence
- Gives either blunt or sticky ends.
- Simpler, no requirement of ATP
- Catalyze the hydrolytic cleavage of phosphodiester bonds in DNA



Nomenclature (How to call particular enzyme?)

- 1st letter - Genus of the bacteria
(From where it is isolated?)
- Next 2-3 letters - Species
- Next letter - Strain
- Roman numerical - Order of discovery
- Example

ECORI - *Escherichia coli*, strain RY - 13

BamHI - *Bacillus amyloliquefaciens*, strain H, I
↓ First (I) restriction endonuclease characterized

Hind III - *Haemophilus influenzae*, strain Rd - I

(Palindromic \rightarrow same from both side)

II. Restriction site / Recognition sequence:

✓ 4/6/8 base pairs

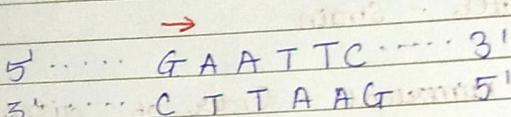
4,096

✓ Two fold axis of rotational symmetry.
(Same direction from both side)

✓ Palindromic in nature

G G A T C C

$$n = 6 \quad 4^6 = 4096$$



5' G A T C 3'

III Frequency of occurrence. - 1/4ⁿ

$$n = \text{No. of base pair}$$

Eg- $n = 4$ $4^n = 256$.
after 256 base pair

North I $n = 8$ base pair.

$$4^8 = 65,536$$

↳ That particular DNA enzyme comes after 65,536 base pair.

Result:- Recognition Sequence for HpaII occurs on average once every 256 bp

Example:- Consider DNA with 50% GC content

The restriction enzyme HpaII recognizes the sequence

5' - G G C C - 3'

3' - C C G G - 5'

The probability of this sequence occurring in DNA is computed as

1st nucleotide pair

G probability = 1/4
C probability = 1/4

2nd nucleotide pair

G probability = 1/4
C probability = 1/4

3rd nucleotide pair

G probability = 1/4
C probability = 1/4

4th nucleotide pair

G probability = 1/4
C probability = 1/4

$$\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = \frac{1}{256}$$

Occurrence of Restriction Sites for Restriction Enzyme in DNA with Randomly Distributed Nucleotide Pairs

Nucleotide Pairs in Restriction site

Occurrence Probability of ~

4

$$(\frac{1}{4})^4 = 1 \text{ in } 256 \text{ bp}$$

5

$$(\frac{1}{4})^5 = 1 \text{ in } 1,024 \text{ bp}$$

6

$$(\frac{1}{4})^6 = 1 \text{ in } 4,096 \text{ bp}$$

8

$$(\frac{1}{4})^8 = 1 \text{ in } 65,476 \text{ bp}$$

n

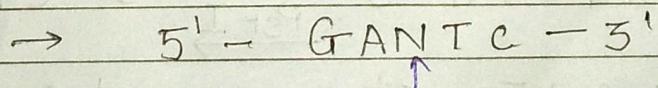
$$(\frac{1}{4})^n$$

IV Classification

- Frequent cutter : 4 or 6 base pair
 - Frequently
- Rare cutter : 8 base pair
 - Rarely (Rare case)

I Degenerative RS (RS site)

- Hinf I → recognizes 5' - GATC - 3'
 - recognizes 4 base pair
 - 4 different type of restriction site
 - 5' - GANTC - 3'
 - GATC
 - C TAG
 - A
- Hinf I → There are certain Restriction enzyme whose Restrictⁿ site is degenerative in nature.



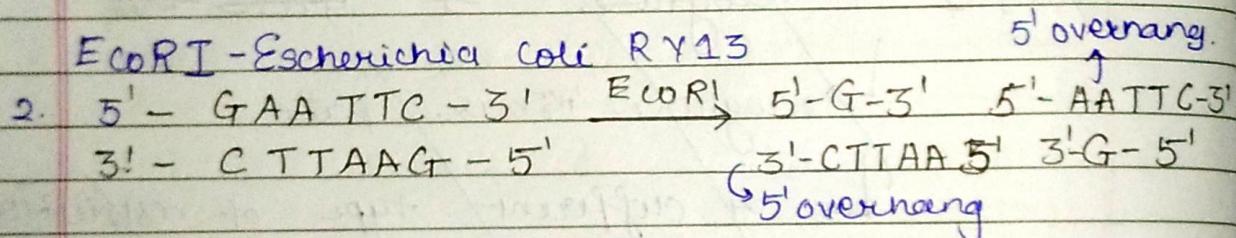
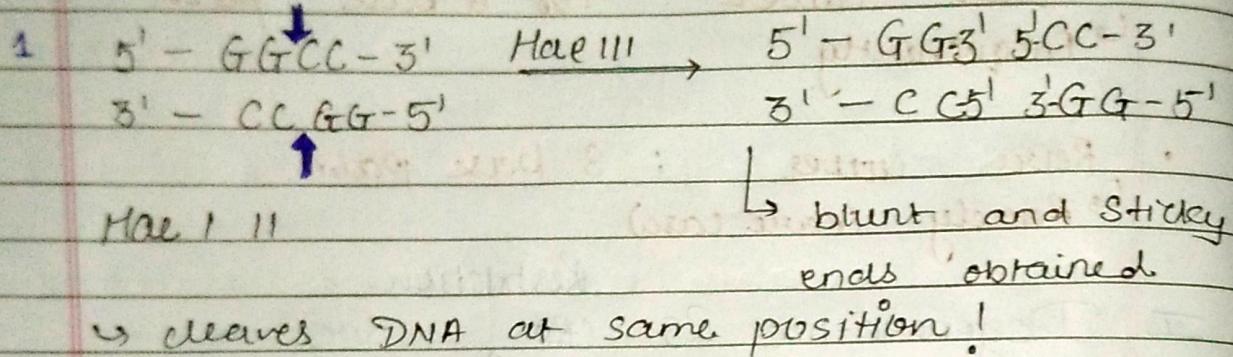
N = maybe any nitrogen bp

∴ Total 4 different types of Restriction site.

like ① GAATC ② GAGTC ③ GATTC
 ④ GACTC

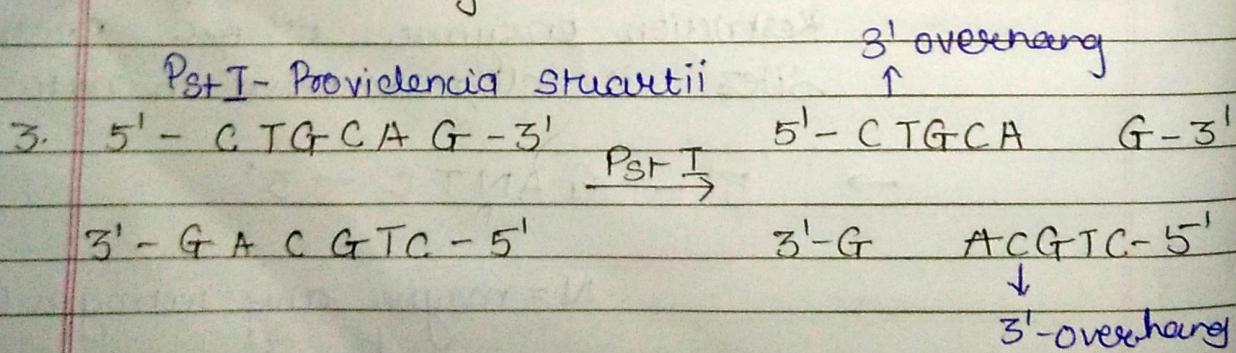
Mode of Action

Hae III - Haemophilus ducrey Phicus



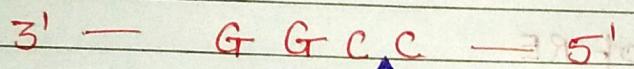
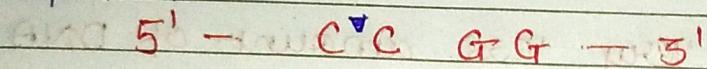
\hookrightarrow Restriction enzyme acts on both strand of DNA but it cleaves at 2 different site gives sticky and blunt end

\hookrightarrow $5'$ overhang



Isoschizomers

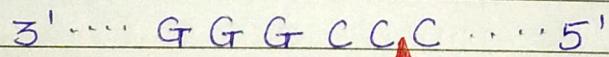
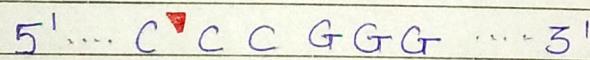
- pair of restriction enzyme obtain from two completely different genera and species but it recognises the same site of cleavage and cleaves it with same manner
- MspI / HpaII



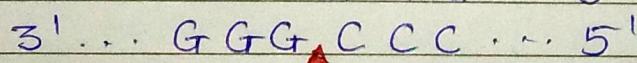
Neoschizomers

- Different species
- Enzyme recognises same restriction site.
- cleavage happens at different position
- In different manner

XbaI



SmaI



Star activity

→ When Type II shows relax (not working properly)

Reasons are

- 1 Use of non ideal strength buffer
- 2 Excess or very less amount of DNA
- 3 Excess of RE
- 4 High glycerol concentration
- 5 Ethanol, DMSO, Phenol contamination
- 6 Prolonged incubation time
- 7 Non optimum temperature

Linker, Adaptor, Homopolymeric
Tailing
&
Terminal Transferase



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Introduction



- Ligation efficiency depends on the ends of DNA in the reaction.
- Mainly two types of end.
 1. “sticky” ends:
 - Ligation is efficient
 - annealing of complementary overhangs brings 5’P and 3’OH into close proximity.
 2. “Blunt” ends:
 - Ligation is less efficient
 - need high concentrations of ligase and DNA

Blunt end ligation

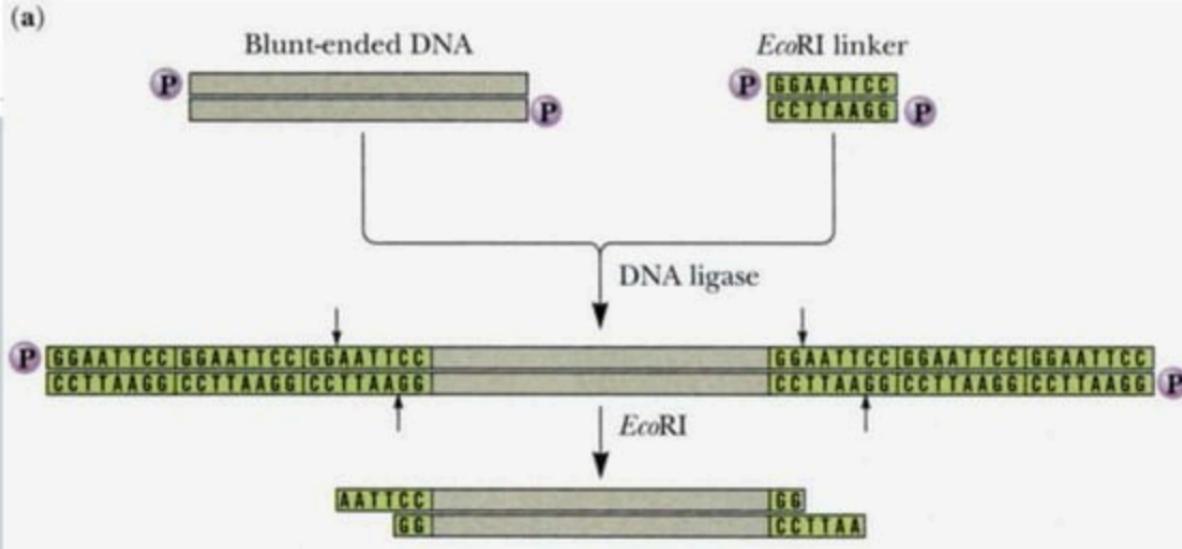


- Mainly **three** methods can be used to put the correct sticky ends onto the DNA fragments-
 1. Cloning foreign DNA by adding **linkers**
 2. Cloning foreign DNA by adding **adaptors**
 3. **Homopolymeric tail** adding by using **Terminal transferase** enzyme.

1. Linker



- Linkers are the **chemically synthesized double stranded DNA oligonucleotides** containing on it **one or more restriction sites** for cleavage by restriction enzymes, e.g. Eco RI, Hind III, Bam HI, etc.
- Linkers are ligated to blunt end DNA by using DNA ligase.
- Both the vector and DNA are treated with restriction enzyme to develop sticky ends.
- The staggered cuts i.e. sticky ends are then ligated with T4 DNA ligase with very high efficiency to the termini of the vector and recombinant plasmid DNA molecules are produced.



Before cloning of a particular sequence in PCR, a primer, associated with a linker is used. This type of primer is called as **linker-primer**. Now-a-days, **two different linkers** are used which has **different RE sites** with F/R primer. This strategy helps in **directional cloning**.

Limitations

- It may be the case that the restriction enzyme used to generate the cohesive ends in the linker **will also cut the foreign DNA at internal sites.**

Solution: CHOOSE ANOTHER RESTRICTION ENZYME

But there may not be a suitable choice if the foreign DNA is large and has sites for several restriction enzymes.

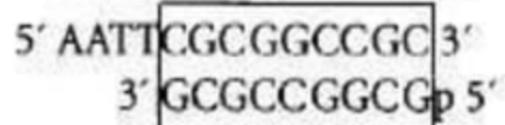
Methylation of internal restriction sites with the Appropriate modification methylase for example EcoRI methylase.

2. Adaptors

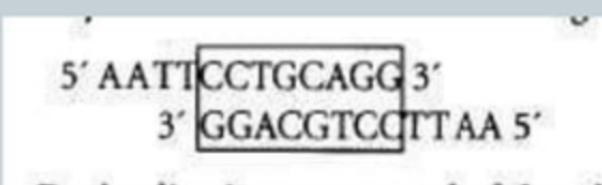


- They are also short double stranded oligonucleotides that carry an **internal RE sites** and **single stranded tails** at one or both ends.
- This protruding sequences can be ligated to DNA fragments containing a **complementary single stranded terminus**.
- After ligation, the DNA can be cleaved with appropriate RE to **create new protruding terminus**.

- Adaptors are available in two basic designs and a variety of specifications.
- 1. Some consists of a partial duplex formed between two oligonucleotides of different length; for example, the **EcoR1-Not1 adaptor**.



- **2.** Another class of adaptor is supplied as an unphosphorylated single oligonucleotide whose sequence is partially self complementary. As an example- **EcoR1-Pst1 adaptor**.

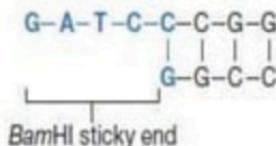


The diagram illustrates the EcoR1-Pst1 adaptor sequence as a hairpin loop. The sequence is shown in two orientations: 5' AATT CCTGCAGG 3' (top) and 3' GGACGTCC TTAA 5' (bottom). The central region, CCTGCAGG, is enclosed in a rectangular box, indicating it is double-stranded and forms the loop of the hairpin. The flanking regions, AATT and GGACGTCC TTAA, are single-stranded.

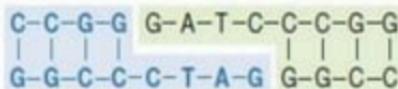
Problems

- The sticky ends of individual adaptors could base pair with themselves to form dimers and the new DNA molecule remains blunt-ended.

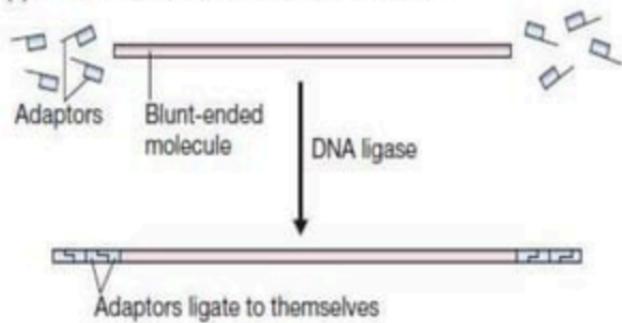
(a) A typical adaptor



(b) Adaptors could ligate to one another



(c) The new DNA molecule is still blunt-ended



Solution

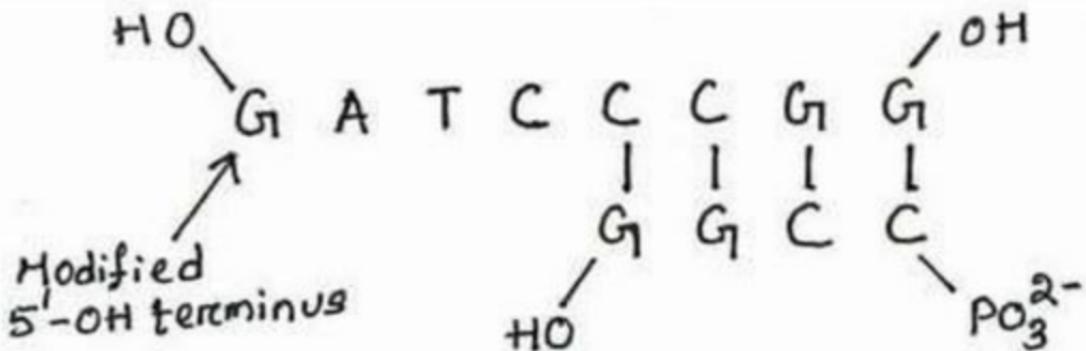


Figure 7: Structure of an adaptor showing the modified 5'-OH terminus

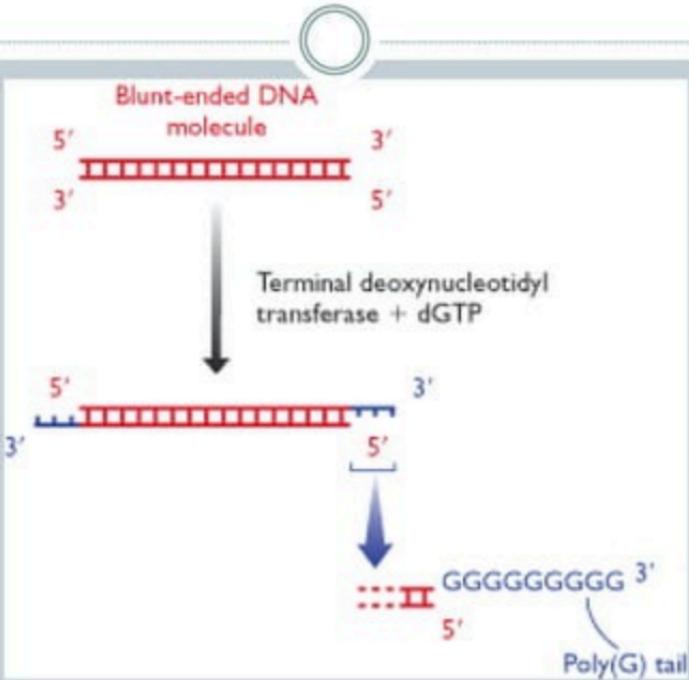
- Adaptor molecules alter their 5' terminus(From 5'-P to 5'-OH) by an enzymatic treatment of the enzyme **Alkaline phosphatase** to prevent self ligation.
- Afterwards, they can be treated with **Polynucleotide kinases** to restore it & ligate to vectors.

- However, H-Bonds can form between the complimentary bases of 2 adaptor.
- The adaptor molecules can be heated **at 90°C for 3 minutes** before use.
- 30X more adaptor is given (in terms of molar) in reaction than insert to ensure proper binding.
- This strategy **eliminates the need of methylation** of cDNA or to digest it with RE before insertion into vector.

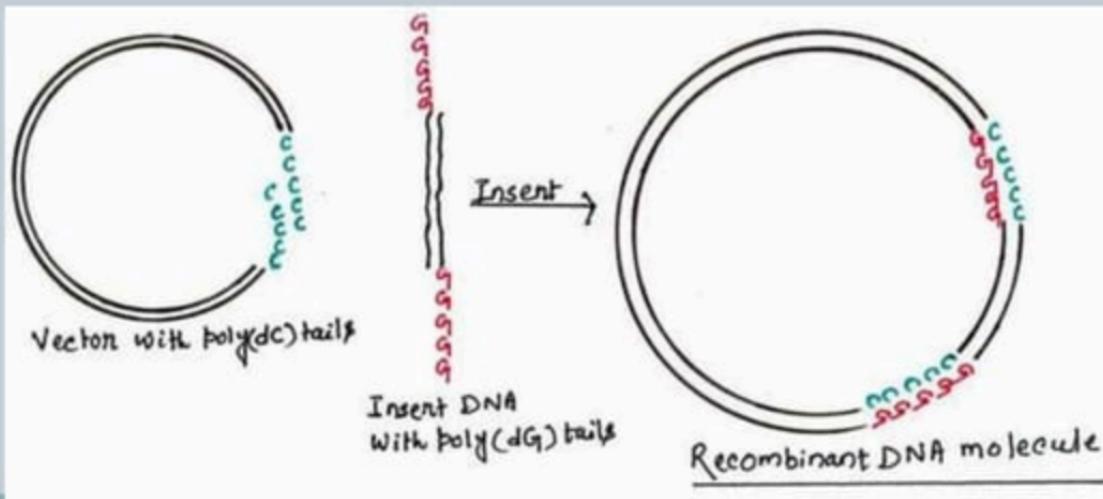
3. Homopolymeric tailing



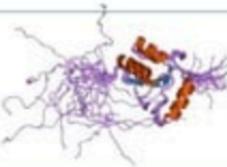
- It is a technique by which **sticky ends** can be produced on a **blunt-ended DNA molecule**.
- In a homopolymer, all the subunits are same. A DNA strand made up **entirely of deoxyguanosine** is an example of homopolymer, and is referred to as **polydeoxyguanosine** or **poly(dG)**.
- Tailing involves using the enzyme **terminal deoxynucleotidyl transferase** to add a series of nucleotides **on to the 3'-OH termini** of a double-stranded DNA molecule.
- The reaction when carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced.



- For ligation of two tailed molecules, **the homopolymers must be complementary**. Frequently poly(dc) tails are attached to the vector and poly(dg) to the DNA to be cloned.



Terminal transferase



- Terminal deoxynucleotidyl transferase (TdT) is a template independent DNA polymerase.
- It is expressed in immature, pre-B, pre-T lymphoid cells, and acute lymphoblastic leukemia/lymphoma cells.
- In humans, terminal transferase is encoded by the *DNTT* gene.

Function

- TdT catalyses the **addition of nucleotides** to the 3' terminus of a DNA molecule. Unlike most DNA polymerases, it **does not require a template**.
- The preferred substrate of this enzyme is a **3'-overhang** but it can also add nucleotides to blunt or recessed 3' ends.
- **Cobalt** is a necessary cofactor, however the enzyme catalyzes reaction upon Mg and Mn administration *in vitro*.

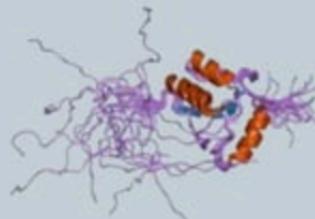
Regulation

- TdT is expressed mostly in the primary lymphoid organs, like the **thymus and bone marrow**.
- Regulation of its expression occurs via multiple pathways. These include protein-protein interactions, like those with **TdIF1**.
- TdIF1 is another protein that interacts with TdT to inhibit its function by masking the DNA binding region of the TdT polymerase

Structure



- Monomeric
- Mol. Wt.- 58000 Da
- Amino acids- 508 to 529(depending upon source)
- A high degree of **sequence homology**(>80%)in TdT between different species



Reaction buffer



- Activity is strongly inhibited by the ammonium ion as well as chloride, iodide and phosphate anions.
- Potassium or sodium cacodylate(dimethyl arsenic acid) buffers are preferred- shown to be optimal for polypurine and polypyrimidine synthesis
- Certain drawbacks with cacodylate such as toxicity, contamination by metal etc

Divalent cation



- Polymerization requires presence of divalent cations.
- Order of efficiency for damp addition to oligonucleotide is as following

Mg>Zn>Co>Mn

- For dGTP- Magnesium ion
- For pyrimidine- Cobalt ion

**Heat Inactivation**

75°C for 20 min

5' - 3' Exonuclease

No

3' - 5' Exonuclease

No

Source

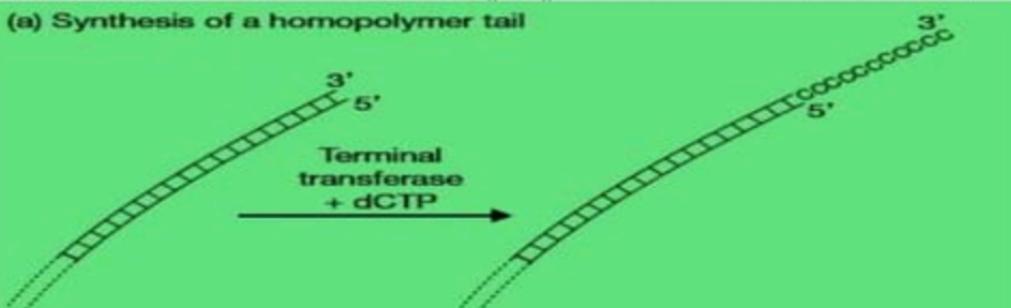
An *E. coli* strain that carries the cloned Terminal Transferase gene from calf thymus.

Applications

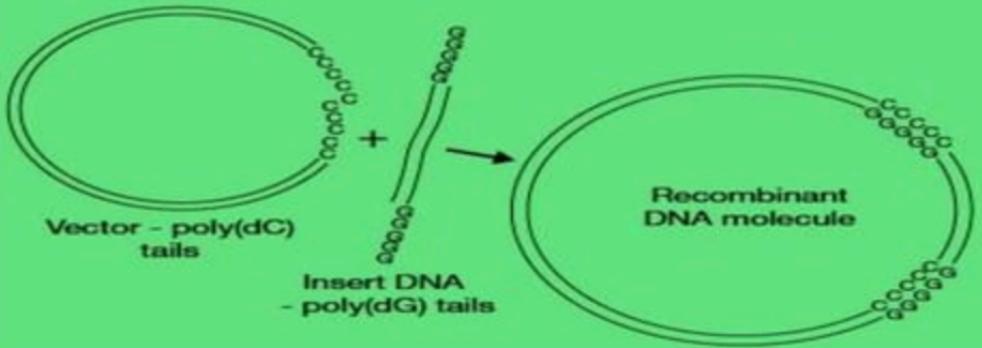


- Addition of homopolymeric tails to plasmid DNA and to cDNA.
- Double- or single-stranded DNA 3'-termini labeling with radioactively labeled or non-radioactively labeled nucleotides.
- Addition of single nucleotides to the 3' ends of DNA for *in vitro* mutagenesis.
- Production of synthetic homo- and heteropolymers.
- RACE (Rapid Amplification of cDNA Ends).
- TUNEL assay (*in situ* localization of apoptosis).

(a) Synthesis of a homopolymer tail



(b) Ligation of homopolymer tails



THANK YOU